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(54) Title:  $\beta$ -SECRETASE, ANTIBODIES TO  $\beta$ -SECRETASE, AND ASSAYS FOR DETECTING  $\beta$ -SECRETASE INHIBITION

## (57) Abstract

Compositions comprising a novel protease capable of cleaving  $\beta$ -amyloid precursor protein (APP) on the amino-terminal side of the  $\beta$ -amyloid peptide therein are provided. The protease is designated  $\beta$ -secretase. Reaction systems comprising  $\beta$ -secretase may be used in screening assays to monitor  $\beta$ -secretase modulated cleavage of APP and to identify  $\beta$ -secretase inhibitors, wherein the  $\beta$ -secretase is in the presence of a suitable polypeptide substrate and cleavage of the substrate is determined in the presence and absence of the test substance. Antibodies are raised against peptides of  $\beta$ -secretase. Pharmaceutical compositions and methods comprise compounds identified by screening assays.

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5         **$\beta$ -SECRETASE, ANTIBODIES TO  $\beta$ -SECRETASE, AND ASSAYS  
FOR DETECTING  $\beta$ -SECRETASE INHIBITION**

**BACKGROUND OF THE INVENTION**

1.    Field of the Invention

10            The present invention relates generally to the  
cleavage of  $\beta$ -amyloid precursor protein to produce  $\beta$ -amyloid  
peptide. More particularly, the present invention relates to  
isolated and purified compositions containing an enzyme  
responsible for such cleavage ( $\beta$ -secretase) and assays for  
15 identifying inhibitors of  $\beta$ -secretase.

Alzheimer's disease is characterized by the presence  
of numerous amyloid plaques and neurofibrillary tangles  
(highly insoluble protein aggregates) present in the brains of  
Alzheimer's disease patients, particularly in those regions  
20 involved with memory and cognition.  $\beta$ -amyloid peptide is a  
major constituent of amyloid plaque which is produced by  
cleavage of  $\beta$ -amyloid precursor protein. It is presently  
believed that a normal (non-pathogenic) processing of the  
 $\beta$ -amyloid precursor protein occurs via cleavage by a putative  
25 " $\alpha$ -secretase" which cleaves between amino acids 16 and 17 of  
the  $\beta$ -amyloid peptide region within the protein. It is  
further believed that pathogenic processing occurs in part via  
a putative " $\beta$ -secretase" which cleaves at the amino-terminus  
of the  $\beta$ -amyloid peptide region within the precursor protein.  
30 Heretofore, however, the existence of  $\beta$ -secretase has not been  
confirmed.

The identification, isolation, and characterization  
of novel biological molecules having unique activities is  
generally useful. For example, novel enzymes can be used to  
35 catalyze reactions of a type associated with their class. In  
particular, novel proteases can be used to cleave proteins for  
a variety of purposes, and the availability of new proteases  
provides unique capabilities. In addition to such uses  
associated with enzymes in general, the identification,

isolation, and purification of the putative  $\beta$ -secretase enzyme would permit chemical modeling of a critical event in the pathology of Alzheimer's disease and would allow the screening of compounds to determine their ability to inhibit  $\beta$ -secretase activity.

For these reasons, it would be desirable to isolate, purify, and characterize the enzyme responsible for the pathogenic cleavage of  $\beta$ -amyloid precursor protein at the amino-terminus of the  $\beta$ -amyloid peptide region. In particular, it would be desirable to utilize such an enzyme (referred to hereinafter as  $\beta$ -secretase) in methods for screening candidate drugs for the ability to inhibit the activity of  $\beta$ -secretase in *in vitro* systems. It would be particularly desirable if such screening assays could be performed in a rapid format which would permit the screening of large numbers of test drugs in automated fashion.

## 2. Description of the Background Art

$\beta$ -amyloid precursor protein (APP) is expressed in three differently-spliced forms of 695, 751, and 770 amino acids, and "normal" processing involves proteolytic cleavage at a site between residues Lys<sup>16</sup> and Leu<sup>17</sup> in the  $\beta$ -amyloid peptide. Kang et al. (1987) *Nature* 325:773-776. Soluble  $\beta$ -amyloid peptide which has been cleaved at the putative  $\beta$ -secretase site has also been found in the culture medium of non-diseased cells (Haass et al. (1992) *Nature* 359:322-325) and in CSF from healthy humans and animals (Seubert et al. (1992) *Nature* 359:325-327). The possible existence of the putative  $\beta$ -secretase is discussed in, for example, Selkoe, "Cell Biology of the Amyloid  $\beta$ -Protein and the Mechanism of Alzheimer's Disease," in *Annual Review of Cell Biology*, Spudich et al., eds., Annual Review, Inc., Palo Alto, California, vol. 10, 1994. The Swedish mutation of APP is also discussed in Selkoe, *supra*. See also, Esch et al. (1994) *Science* 248:1122.

## SUMMARY OF THE INVENTION

The present invention provides novel  $\beta$ -secretase compositions comprising an isolated and purified enzyme which cleaves  $\beta$ -amyloid precursor protein (APP) at the amino-terminus of  $\beta$ -amyloid peptide ( $\beta$ AP) within APP, referred to hereinafter as " $\beta$ -secretase activity." The compositions of the present invention will generally have a  $\beta$ -secretase activity which is at least five-fold greater than that of a solubilized but unenriched membrane fraction from human 293 cells, preferably being at least ten-fold greater than that of the membrane fraction, and more preferably being at least 100-fold greater than that of the membrane fraction. The  $\beta$ -secretase enzyme is characterized by (1) an apparent molecular weight in the range from 260 kD to 300 kD as determined by gel exclusion chromatography, (2) a more accurate apparent molecular weight in the range from 60 kD to 148 kD determined by electrophoresis, (3) a net negative charge at pH 5 and a net negative charge at pH 7.5, and (4) binding to wheat germ agglutinin.

The compositions of the present invention are generally useful as proteolytic chemicals and specifically useful in assays for detecting proteolytic cleavage of APP resulting from the novel  $\beta$ -secretase and determining whether a test substance will inhibit such cleavage. The method comprises exposing a polypeptide comprising the  $\beta$ -secretase site of APP (located at the amino-terminus of the  $\beta$ AP region within APP) to an at least partially purified  $\beta$ -secretase in the presence of the test substance under conditions such that the  $\beta$ -secretase would be expected to cleave the polypeptide into an amino-terminal fragment and a carboxy-terminal fragment in the absence of test substance which inhibits such cleavage. Test substances which inhibit such cleavage may then be introduced or exposed to the assay system to identify which test substances have  $\beta$ -secretase inhibition activity. Such test methods preferably employ the  $\beta$ -secretase compositions described above. Generation of fragments of APP-derived polypeptides is detected, e.g. by an antibody specific for the carboxy end of the amino terminal fragment or

the amino end of the carboxy-terminal fragment. The polypeptide substrate for the  $\beta$ -secretase may comprise a fusion polypeptide including an amino-terminal portion having a binding epitope. Use of such a fusion polypeptide as the  $\beta$ -secretase substrate facilitates detection of cleavage by capture of the amino-terminal portion and labelling of the amino-terminal portion.

The compositions will further comprise threshold levels, typically at least 10% by weight, of enzymes which cleave APP at the  $\beta$ AP cleavage site and which are reactive with antibodies raised against immunogenic peptides of  $\beta$ -secretase, such as any one or a combination of [SEQ ID No.:5], [SEQ ID No.:6], and [SEQ ID No.:7].

The present invention still further provides antibodies and antibody compositions that specifically bind to  $\beta$ -secretase protein. The antibodies may be polyclonal or monoclonal, and may be prepared by immunization of a suitable host with any of the immunogenic  $\beta$ -secretase compositions described above. The antibodies may further be prepared recombinantly, may be humanized, or otherwise modified or produced in accordance with conventional methods for antibody production.

The present invention further provides methods and assays for detecting  $\beta$ -secretase cleavage of a polypeptide substrate, such as  $\beta$ -amyloid precursor protein (APP) or synthetic or recombinant analogues thereof. The method utilizes a reaction system including  $\beta$ -secretase and the polypeptide substrate present in initial amounts. The reaction system is maintained under conditions which permit the  $\beta$ -secretase to cleave the polypeptide substrate into cleavage products. The  $\beta$ -secretase cleavage reaction is monitored by detecting the amount of at least one of the  $\beta$ -secretase cleavage products, where the amount of cleavage product(s) will increase over time as the reaction progresses. Such methods are particularly useful for screening test compounds for the ability to inhibit  $\beta$ -secretase activity. Test compounds are introduced to the reaction system, and the ability of the test compound to inhibit the  $\beta$ -secretase

activity is determined based on the ability to decrease the amount of cleavage product produced, usually in comparison to a control where  $\beta$ -secretase mediated cleavage in the reaction system is observed and measured in the absence of test compound(s).

The reaction system may comprise  $\beta$ -secretase and polypeptide substrate obtained from separate sources. For example,  $\beta$ -secretase may be purified from a natural source or be synthetically or recombinantly produced, as discussed in detail hereinbelow. In such cases, the polypeptide substrate may be full length APP, but will more usually be a shorter polypeptide comprising the  $\beta$ -secretase cleavage site within APP. The shorter polypeptide can be produced with label, binding moiety, or other components which facilitate detection in various assay protocols.

In an alternative assay format, both the  $\beta$ -secretase and the polypeptide substrate will be obtained from a single cellular source, e.g. cell membranes from brain cells or other suitable sources. The cellular source will be treated to release both the  $\beta$ -secretase and the polypeptide substrate (which will be full length APP) into a suitable reaction medium, where the conversion of APP into cleavage products may be observed over time. Test compounds may be introduced to the reaction system, and the ability of particular test compounds to inhibit  $\beta$ -secretase activity determined generally as described elsewhere herein.

The present invention further comprises methods for inhibiting the cleavage of  $\beta$ -amyloid precursor protein (APP) in cells. Such methods comprise administering to the cells an amount of a compound effective to at least partially inhibit  $\beta$ -secretase activity. Usually, such compounds will be selected by the screening methods described above.

The present invention still further provides methods for inhibiting the cleavage of  $\beta$ -amyloid precursor protein in mammalian hosts. Such methods comprise administering to the host an amount of a compound effective to inhibit  $\beta$ -secretase activity in cells of the host, usually in brain cells of the host. Such compounds will usually be selected by the

screening assays described above. Such methods will be useful for treating conditions related to  $\beta$ -amyloid peptide deposition such as Alzheimer's disease, Down's syndrome, and the like.

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#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a Western blot showing the reactivity of antibodies raised against peptides Seek-1 [SEQ ID No.:5], Seek-2 [SEQ ID No.:6], and Seek-3 [SEQ ID No.:7], under non-reducing conditions, as described in the Experimental section.

Fig. 2 is a similar Western blot to Fig. 8, except that the protein samples were reduced prior to electrophoresis.

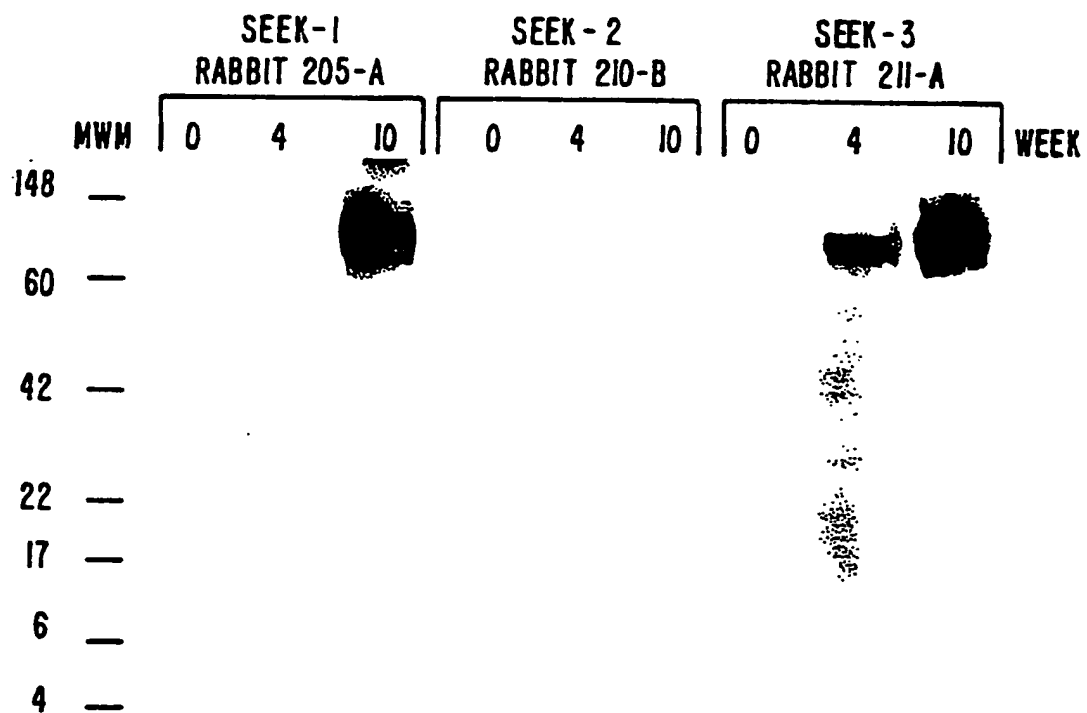
Fig. 3 is a chart comparing the immunoprecipitation of  $\beta$ -secretase using the antibodies of Figs. 8 and 9 under reducing and non-reducing conditions.

Fig. 4 is a schematic illustration of an APP-containing fusion peptide useful as substrates in performing the screening assays of the present invention, having a binding epitope derived from maltose-binding protein (MBP). An assay was run by exposing the fusion polypeptide to  $\beta$ -secretase which cleaves the 125 amino acid portion of APP (APP C-125) at the amino-terminus of the  $\beta$ AP. The MBP portion may then be captured, and the carboxy-terminus of the APP fragment which is exposed by cleavage with  $\beta$ -secretase may be identified with 192 antibody specific for said terminus. SW-192 antibody bound to a reporter is utilized, which antibody recognizes the carboxy-terminus of the Swedish mutation of APP.

Fig. 5 illustrates APP 638 which is a recombinantly expressed form of APP truncated after  $\beta$ AP ( $A\beta$ ). APP 638 may be used in a  $\beta$ -secretase assay where the  $\beta$ AP peptide is cleaved and the carboxy-terminus of the amino-terminal fragment of APP 638 recognized by 192 antibody in either a Western blot or ELISA assay. The carboxy terminal  $\beta$ AP fragment can also be measured using a 3D6/266 assay.



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*FIG. 2.*



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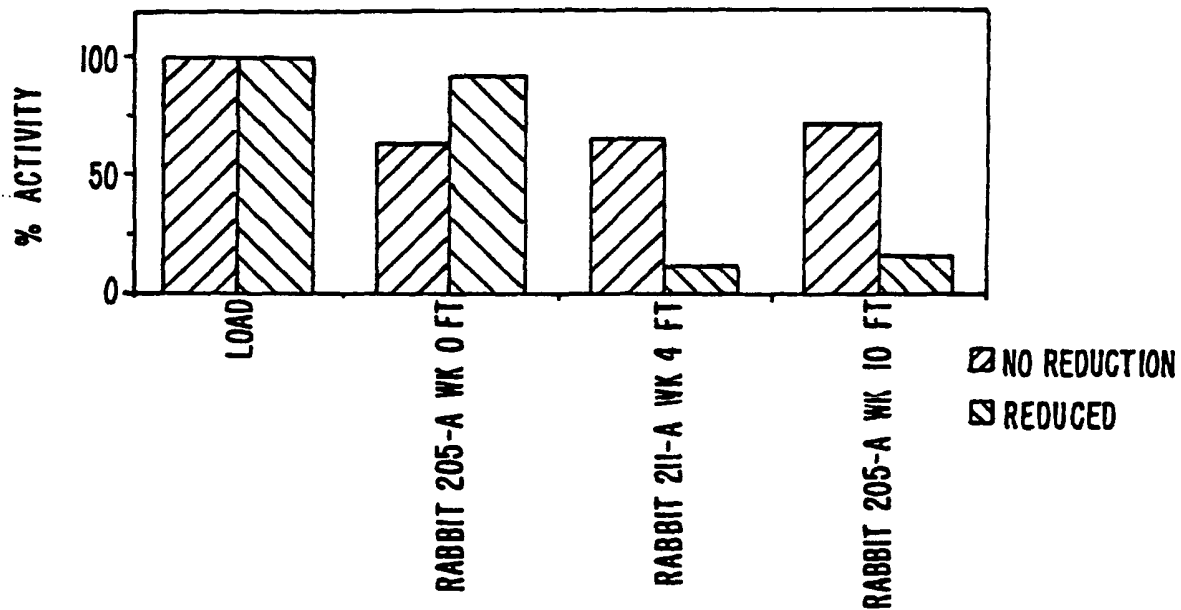


FIG. 3.

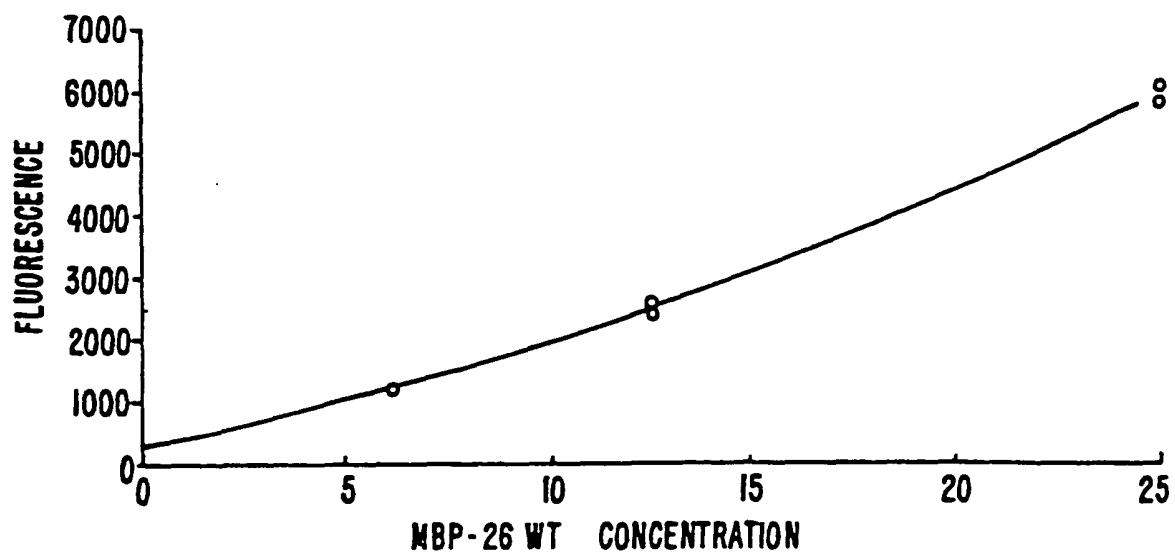


FIG. 8.



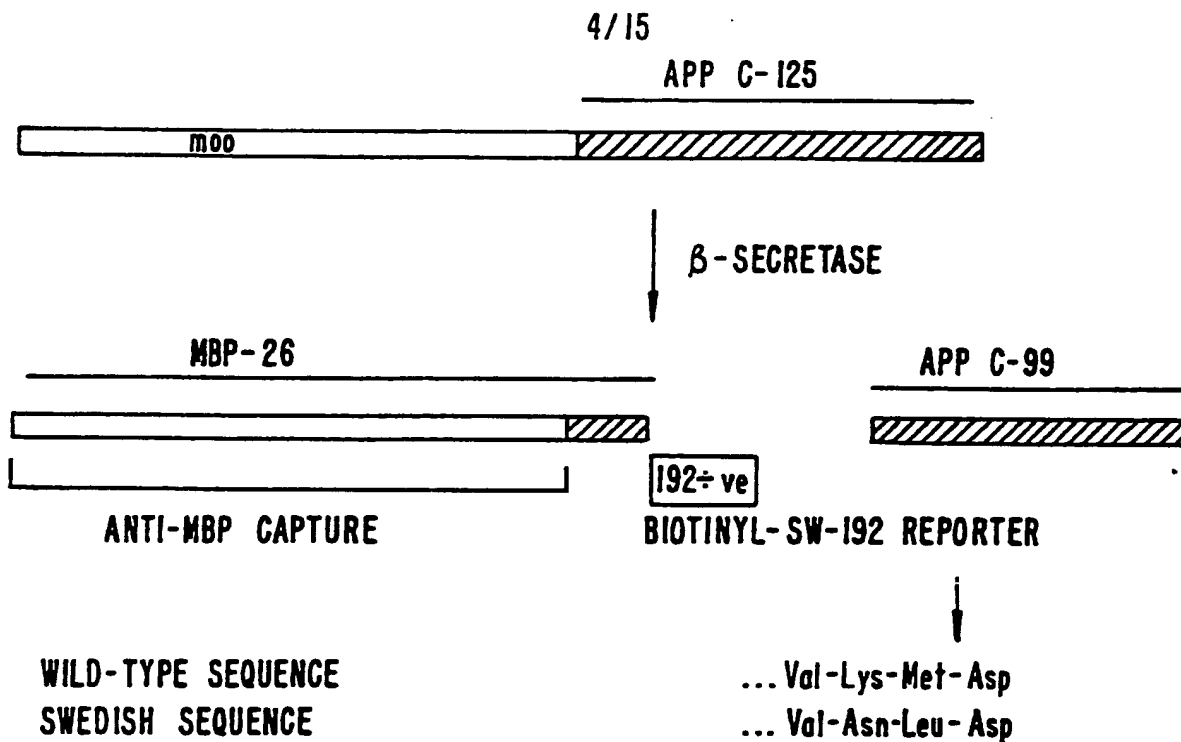


FIG. 4.

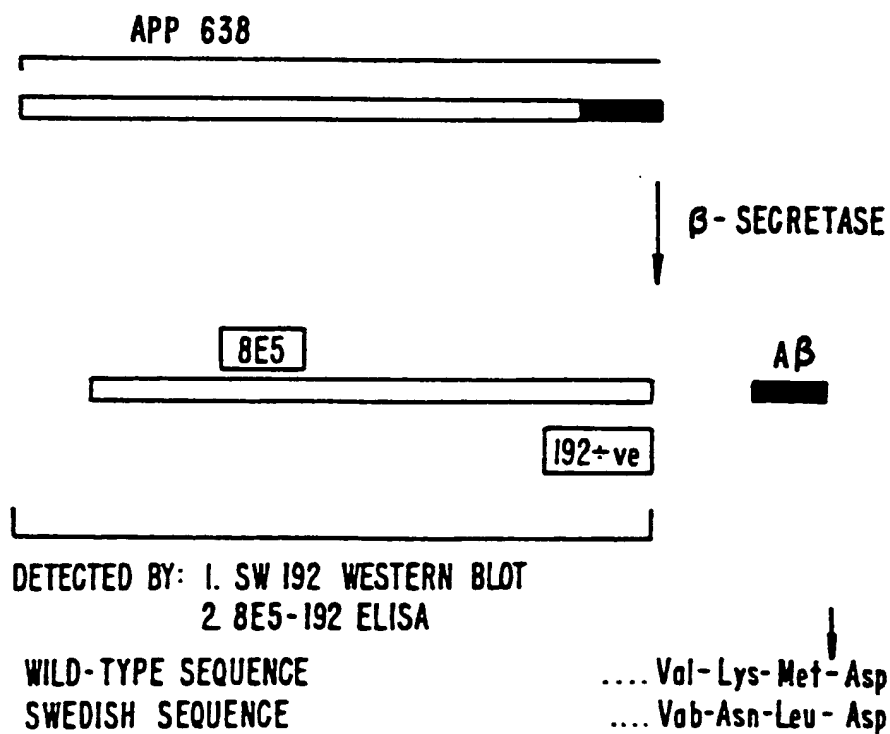


FIG. 5.

SUBSTITUTE SHEET (RULE 26)



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1 ATGAAACTGAAGAAGGTAACTGGTAATCTGGATTAACGGCGATAAAGGC  
1 MetLysThrGluGluGlyLysLeuValIleTrpIleAsnGlyAspLysGly

52 TATAACGGTCTCGCTGAAGTCGGTAAGAAATTCGAGAAAGATACCGGAATT  
18 TyrAsnGlyLeuAlaGluValGlyLysLysPheGluLysAspThrGlyIle

103 AAAGTCACCGTTGAGCATCCGGATAAACTGGAAGAGAAATTCACACAGGTT  
35 LysValThrValGluHisProAspLysLeuGluGluLysPheProGlnVal

154 GCGGCAACTGGCGATGGCCCTGACATTATCTTCTGGGCACACGACCGCTTT  
52 AlaAlaThrGlyAspGlyProAspIleIlePheTrpAlaHisAspArgPhe

205 GGTGGCTACGCTCAATCTGGCCTGTTGGCTGAAATCACCCCGGACAAAGCG  
69 GlyGlyTyrAlaGlnSerGlyLeuLeuAlaGluIleThrProAspLysAla

256 TTCCAGGACAAGCTGTATCCGTTTACCTGGGATGCCGTACGTTACAACGGC  
86 PheGlnAspLysLeuTyrProTheThrTrpAspAlaValArgTyrAsnGly

307 AAGCTGATTGCTTACCCGATCGCTGTTGAAGCGTTATCGCTGATTTATAAC  
103 LysLeuIleAlaTyrProIleAlaValGluAlaLeuSerLeuIleTyrAsn

358 AAAGATCTGCTGCCGAACCCGCCAAAAACCTGGGAAGAGATCCCGGCGCTG  
120 LysAspLeuLeuProAsnProProLysThrTrpGluGluIleProAlaLeu

409 GATAAAGAACTGAAAGCGAAAGGTAAGAGCGCGCTGATGTTCAACCTGCAA  
137 AspLysGluLeuLysAlaLysGlyLysSerAlaLeuMetPheAsnLeuGln

460 GAACCGTACTTCACCTGGCCGCTGATTGCTGCTGACGGGGGTTATGCGTTC  
154 GluProTyrPheThrTrpProLeuIleAlaAlaAspGlyGlyTyrAlaPhe

511 AAGTATGAAAACGGCAAGTACGACATTAAAGACGTGGGCGTGGATAACGCT  
171 LysTyrGluAsnGlyLysTyrAspIleLysAspValGlyValAspAsnAla

562 GGCGCGAAAGCGGGTCTGACCTTCCTGGTTGACCTGATTAAAAACAAACAC  
188 GlyAlaLysAlaGlyLeuThrPheLeuValAspLeuIleLysAsnLysHis

613 ATGAATGCAGACACCGATTACTCCATCGCAGAAGCTGCCTTTAATAAAGGC  
205 MetAsnAlaAspThrAspTyrSerIleAlaGluAlaAlaPheAsnLysGly

664 GAAACAGCGATGACCATCAACGGCCCGTGGGCATGGTCCAACATCGACACC  
222 GluThrAlaMetThrIleAsnGlyProTrpAlaTrpSerAsnIleAspThr

**FIG. 6-1**





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715 AGCAAAGTGAATTATGGTGTAAACGGTACTGCCGACCTTCAAGGGTCAACCA  
239 SerLysValAsnTyrGlyValThrValLeuProThrPheLysGlyGlnPro

766 TCCAAACCGTTCGTTGGCGTGCTGAGCGCAGGTATTAACGCCGCCAGTCCG  
256 SerLysProPheValGlyValLeuSerAlaGlyIleAsnAlaAlaSerPro

817 AACAAAGAGCTGGCGAAAGAGTTCCTCGAAAACCTATCTGCTGACTGATGAA  
273 AsnLysGluLeuAlaLysGluPheLeuGluAsnTyrLeuLeuThrAspGlu

868 GGTCTGGAAGCGGTTAATAAAGACAAACCGCTGGGTGCCGTAGCGCTGAAG  
290 GlyLeuGluAlaValAsnLysAspLysProLeuGlyAlaValAlaLeuLys

919 TCTTACGAGGAAGAGTTGGCGAAAGATCCACGTATTGCCGCCACCATGGAA  
307 SerTyrGluGluGluLeuAlaLysAspProArgIleAlaAlaThrMetGlu

970 AACGCCCAGAAAGGTGAAATCATGCCGAACATCCCGCAGATGTCCGCTTTC  
324 AsnAlaGlnLysGlyGluIleMetProAsnIleProGlnMetSerAlaPhe

1021 TGGTATGCCGTGCGTACTGCGGTGATCAACGCCGCCAGCGGTTCGTCAGACT  
341 TrpTyrAlaValArgThrAlaValIleAsnAlaAlaSerGlyArgGlnThr

1072 GTCGATGAAGCCCTGAAAGACGCGCAGACTAATTCGAGCTCGGTACCCGGC  
358 ValAspGluAlaLeuLysAspAlaGlnThrAsnSerSerSerValProGly

1123 CGGGGATCCATCGAGGGTAGGGCCGACCGAGGACTGACCACTCGACCAGGT  
375 ArgGlySerIleGluGlyArgAlaAspArgGlyLeuThrThrArgProGly

1174 TCTGGGTTGACAAATATCAAGACGGAGGAGATCTCTGAAGTGAATCTGGAT  
392 SerGlyLeuThrAsnIleLysThrGluGluIleSerGluValAsnLeuAsp

1225 GCAGAATTCCGACATGACTCAGGATATGAAGTTCATCATCAAAAATTGGTG  
409 AlaGluPheArgHisAspSerGlyTyrGluValHisHisGlnLysLeuVal

1276 TTCTTTGCAGAAGATGTGGGTTCAAACAAAGGTGCAATCATTGGACTCATG  
426 PhePheAlaGluAspValGlySerAsnLysGlyAlaIleIleGlyLeuMet

1327 GTGGGCGGTGTTGTCATAGCGACAGTGATCGTCATCACCTTGGTGATGCTG  
443 ValGlyGlyValValIleAlaThrValIleValIleThrLeuValMetLeu

1378 AAGAAGAAACAGTACACATCCATTCATCATGGTGTGGTGGAGGTTGACGCC  
460 LysLysLysGlnTyrThrSerIleHisHisGlyValValGluValAspAla

**FIG. 6-2**



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1429 GCTGTCACCCCAGAGGAGCGCCACCTGTCCAAGATGCAGCAGAACGGCTAC

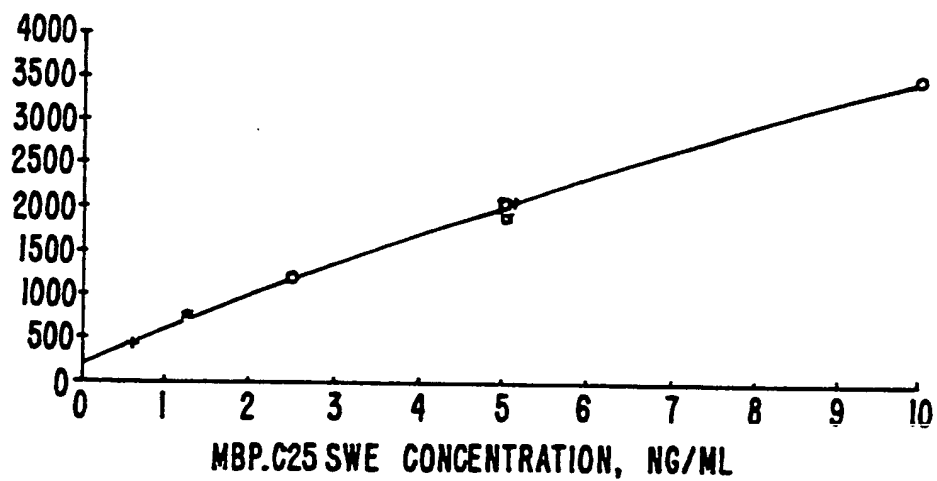
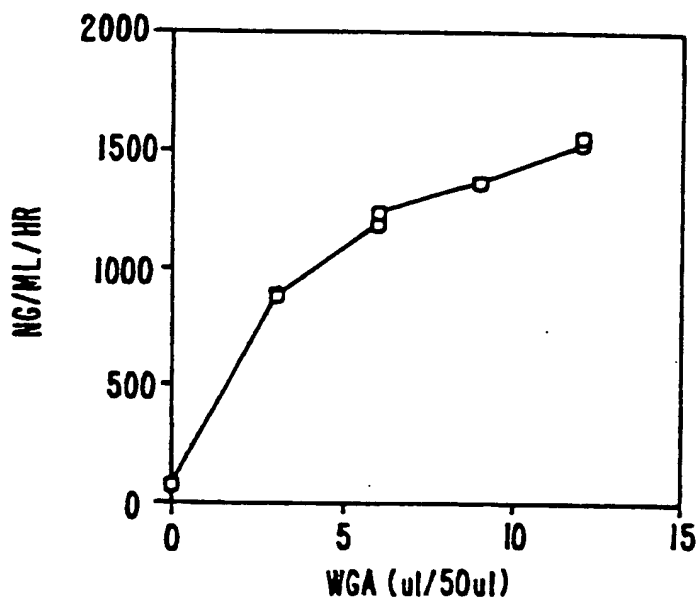
477 AlaValThrProGluGluArgHisLeuSerLysMetGlnGlnAsnGlyTyr

1480 GAAAATCCAACCTACAAGTTCTTTGAGCAGATGCAGAACTAG

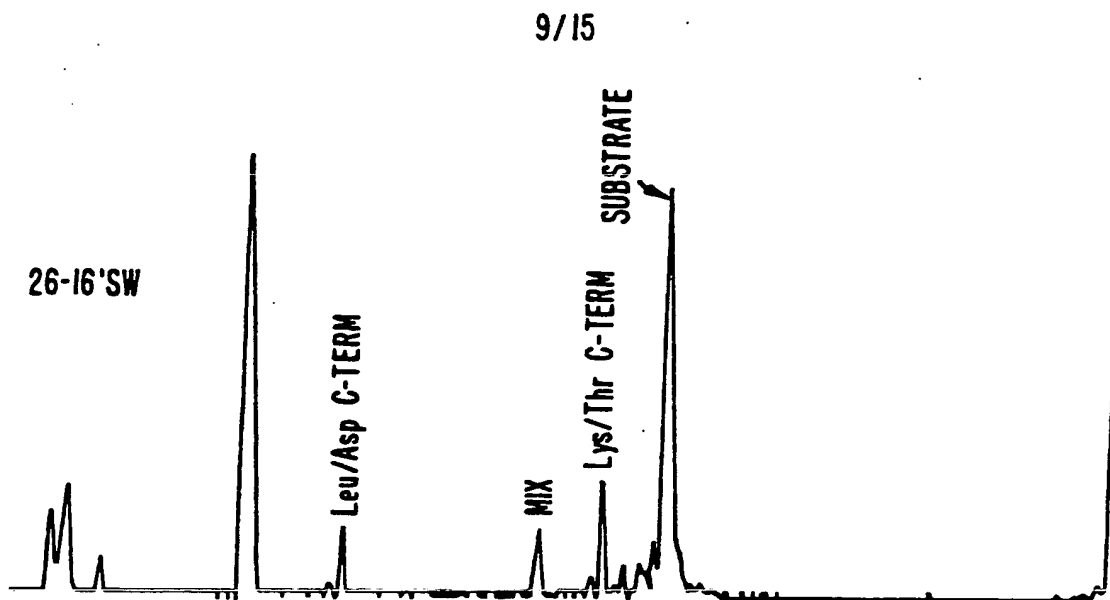
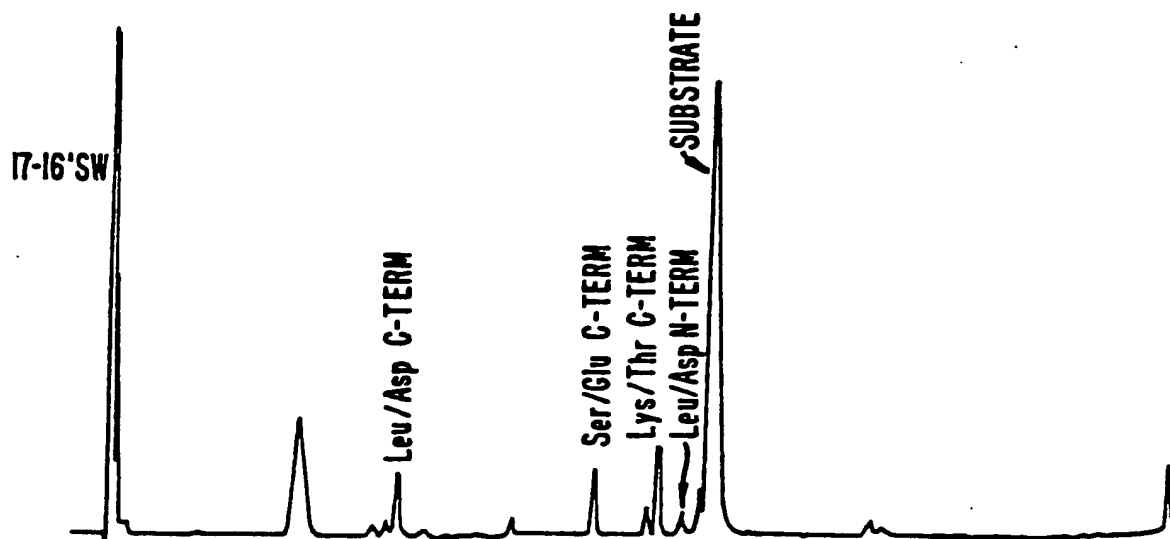
494 GluAsnProThrTyrLysPhePheGluGlnMetGlnAsn...**FIG. 6-3**



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*FIG. 7.**FIG. 9.*



*FIG. 10A.**FIG. 10B.*





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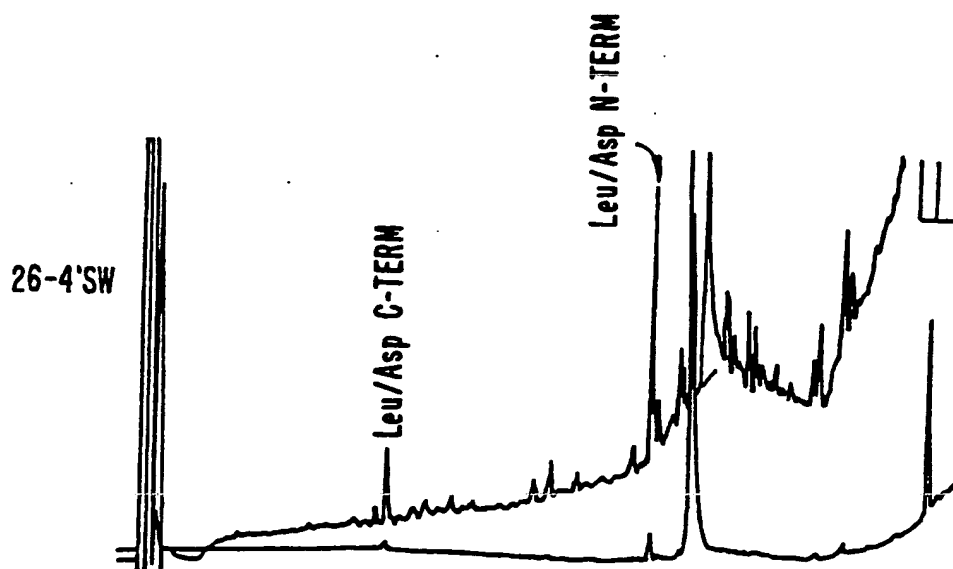


FIG. 10C.

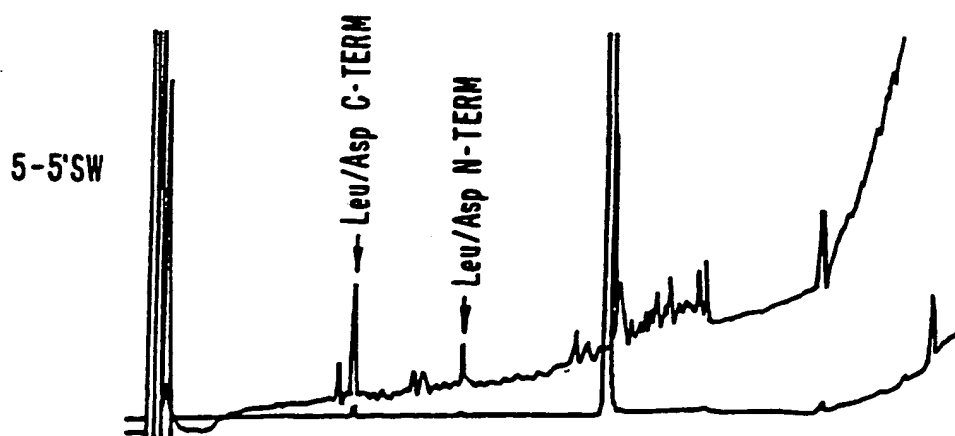


FIG. 10D.



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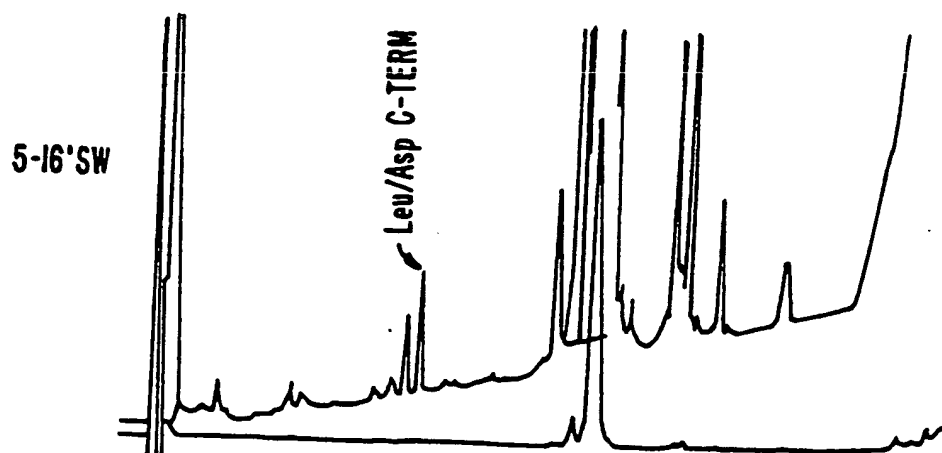


FIG. 10E.

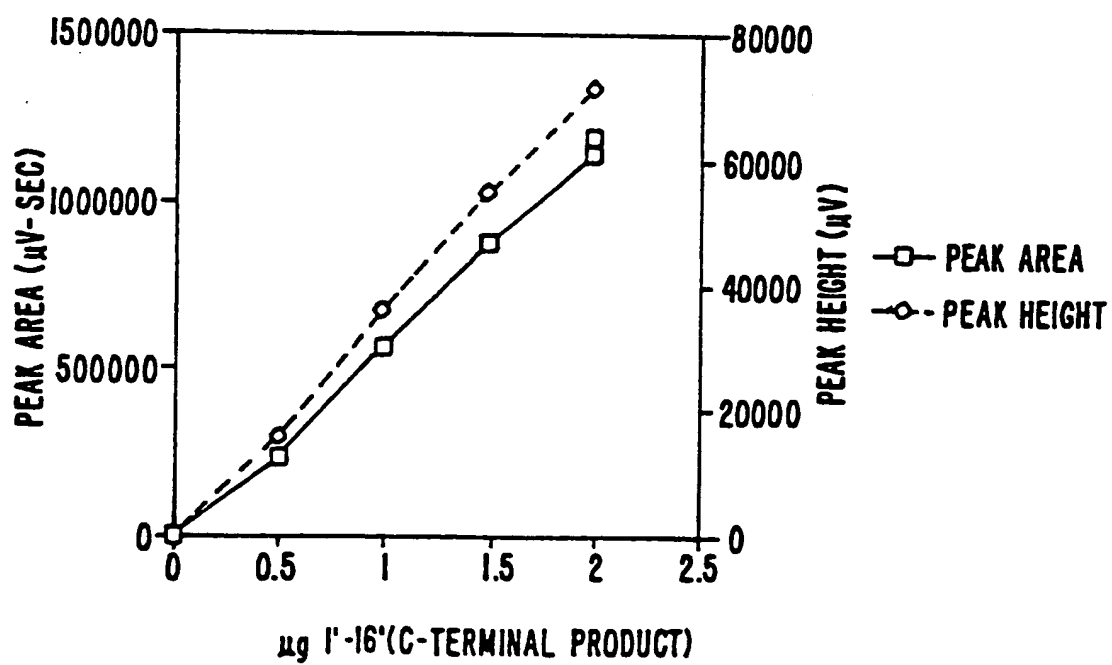


FIG. 10F.



v

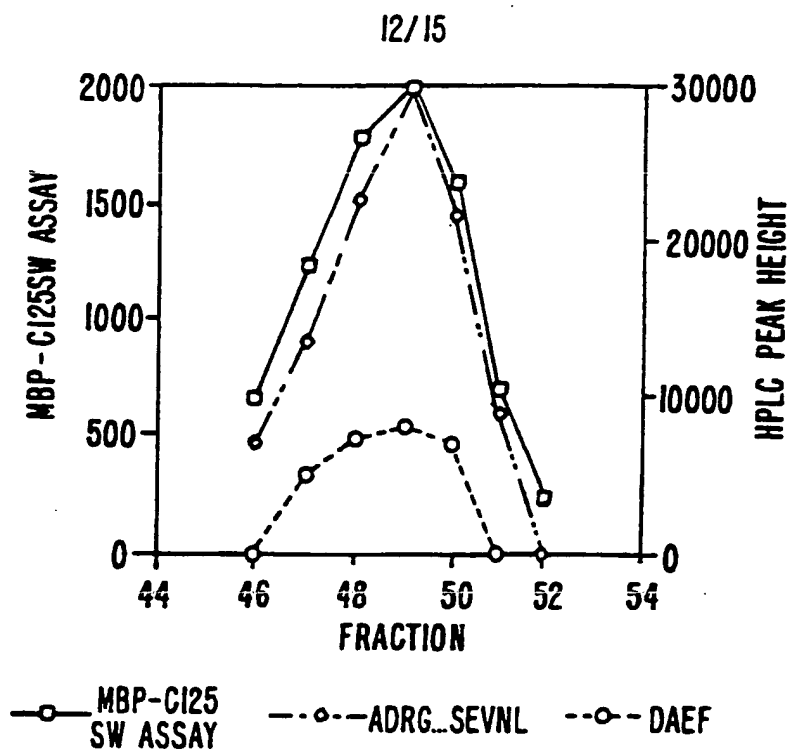


FIG. IIA.

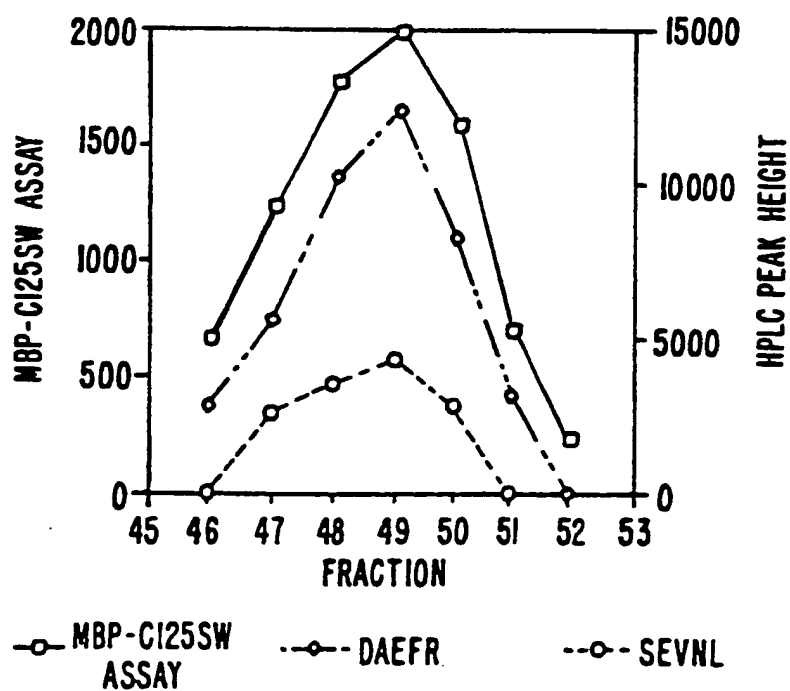


FIG. IIB.



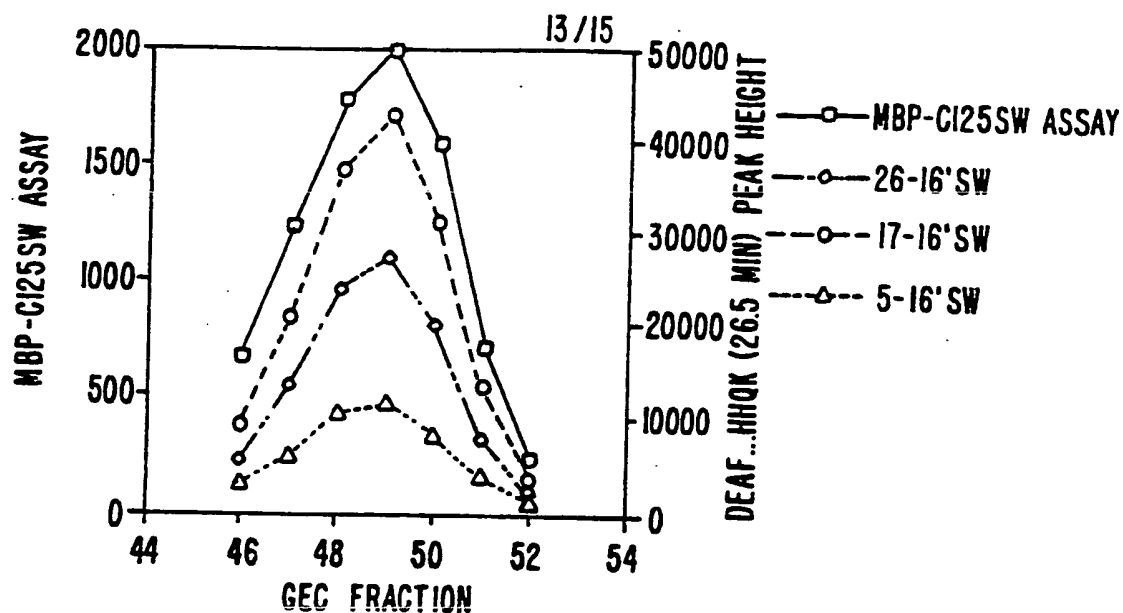


FIG. 11C.

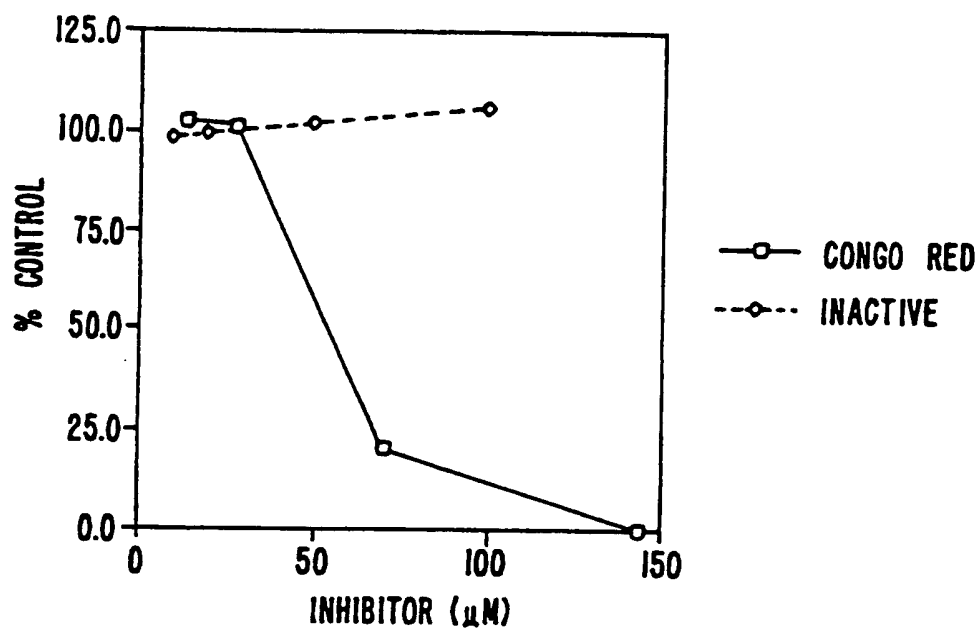


FIG. 12.

STANDARD 0 — 0 22562 22863 22866 0 - ADDITIONS

0 45 100 — 180 - MINUTES INCUBATED

— — — — —

5' 15'





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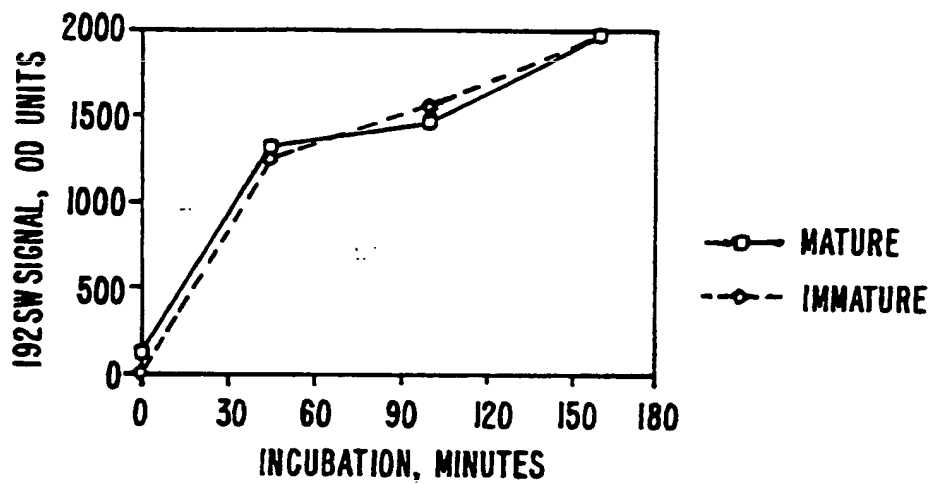


FIG. 14.

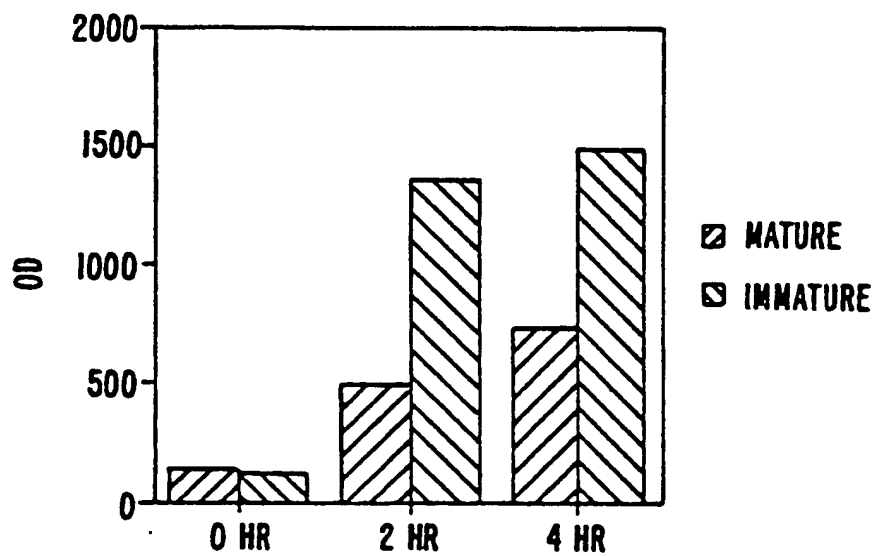
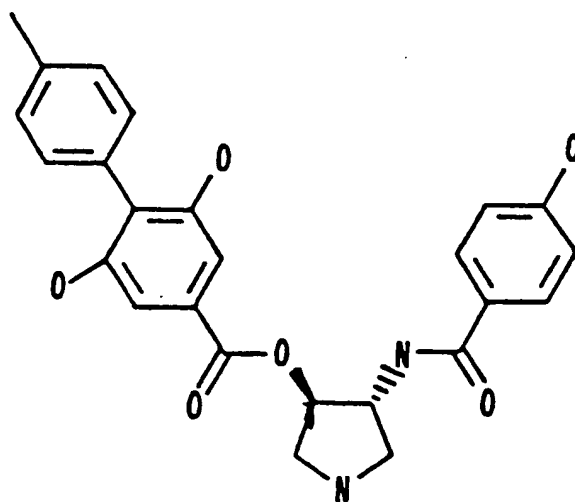
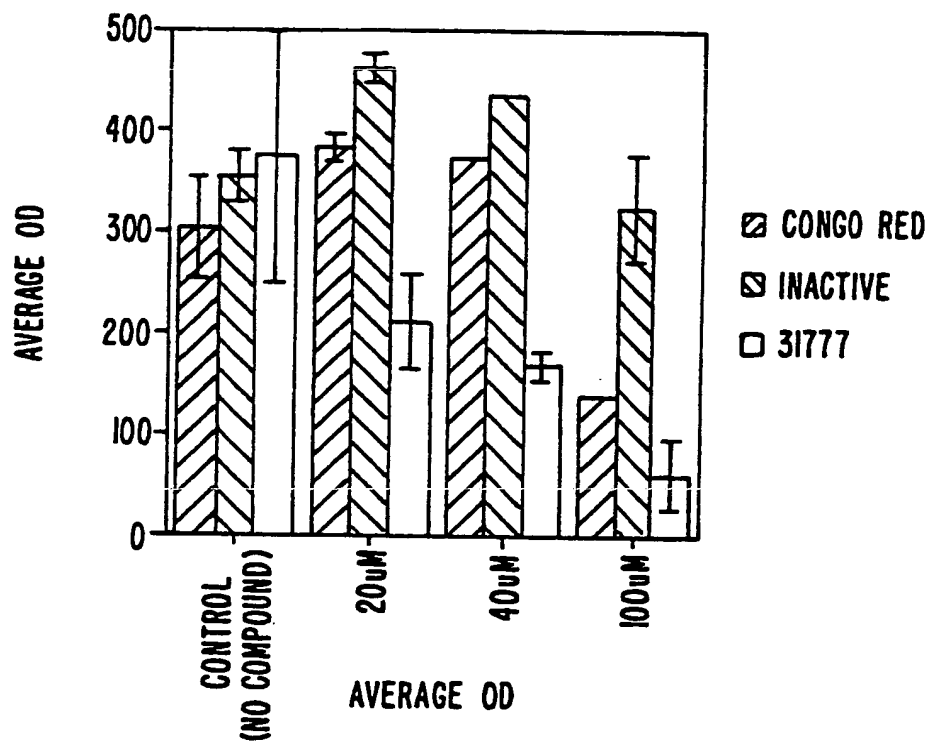


FIG. 15.



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**FIG. 17.**

